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The Adaptor CARD9 Is Required for Adaptive but Not Innate Immunity to Oral Mucosal *Candida albicans* Infections

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Oropharyngeal candidiasis (OPC [thrush]) is an opportunistic infection caused by the commensal fungus *Candida albicans*. OPC is common in individuals with HIV/AIDS, infants, patients on chemotherapy, and individuals with congenital immune defects. Immunity to OPC is strongly dependent on the interleukin-23 (IL-23)/IL-17R axis, as mice and humans with defects in IL-17R signaling (*IL17F*, *ACT1*, *IL-17RA*) or in genes that direct Th17 differentiation (*STAT3*, *STAT1*, *CARD9*) are prone to mucocutaneous candidiasis. Conventional Th17 cells are induced in response to *C. albicans* infection via signals from C-type lectin receptors, which signal through the adaptor CARD9, leading to production of Th17-inducing cytokines such as IL-6, IL-1β, and IL-23. Recent data indicate that IL-17 can also be made by numerous innate cell subsets. These innate "type 17" cells resemble conventional Th17 cells, but they can be activated without need for prior antigen exposure. Because *C. albicans* is not a commensal organism in rodents and mice are thus naive to this fungus, we had the opportunity to assess the role of CARD9 in innate versus adaptive responses using an OPC infection model. As expected, CARD9$^{-/-}$ mice failed to mount an adaptive Th17 response following oral *Candida* infection. Surprisingly, however, CARD9$^{-/-}$ mice had preserved innate IL-17-dependent responses to *Candida* and were almost fully resistant to OPC. Thus, CARD9 is important primarily for adaptive immunity to *C. albicans*, whereas alternate recognition systems appear to be needed for effective innate responses.

Candida albicans is a commensal fungus of the oral cavity, vaginal mucosa, skin, and gastrointestinal (GI) tract. Oropharyngeal candidiasis (OPC [thrush]) is a frequent opportunistic infection of T cell deficiency, particularly in AIDS (1). *C. albicans* also causes invasive infections with high mortality in hospital settings (2). In humans, circulating *Candida*-responsive cells are almost exclusively of the Th17 phenotype (3). Individuals with impaired differentiation of Th17 cells (mutations in *STAT1*, *STAT3*, or *IL12RB*) or defects in interleukin-17A (IL-17A) signaling (mutations in *IL17RA*, *IL17F*, or *ACT1*) are markedly susceptible to chronic mucocutaneous candidiasis (CMC) (4–6).

Host defense against *C. albicans* is initiated by Toll-like receptors (TLRs), inflammasomes, and C-type lectin receptors (CLRs) (7). CLRs are expressed on antigen-presenting cells (APCs) and recognize carbohydrate moieties in the *Candida* cell wall such as β-glucans and mannans. CLRs activate NF-κB via CARD9 (8, 9), leading to induction of Th17-promoting cytokines (IL-1β, IL-6, IL-23) (10). Mice with deficiencies in CLRs (namely, Dectin-1, -2, and -3 and Mincle) or CARD9 are susceptible to disseminated candidiasis, which is associated with reduced Th17 cell frequency (10–13). A family with CARD9 mutations was identified that suffered from both invasive and mucocutaneous candidiasis, which also correlated with low Th17 levels (14). Dectin-1 single nucleotide polymorphisms (SNPs) have also been linked to CMC (15, 16). Thus, generation of adaptive Th17 responses by CLRs and CARD9 appears to be critical for immunity to CMC.

All humans are exposed to *C. albicans* early in life and thus generate strong adaptive responses to this microbe, but relatively little is known about innate responses to *Candida*. Mice, in contrast, are naive to *C. albicans* and do not exhibit cross-reactive adaptive responses to other *Candida* species or yeast derivatives in food (17–19). However, mice mount rapid and powerful IL-17R-dependent responses to oral *C. albicans* infections, indicative of a strong innate response (20, 21). Similarly to humans, mice exposed to *C. albicans* generate long-term adaptive Th17 cell responses that confer additional protection from infection (18, 19). The contribution of CARD9 to innate antifungal responses is not well understood, so we assessed its role in an acute model of OPC. As expected, CARD9 was required for generating protective adaptive Th17 responses to OPC. Surprisingly, however, CARD9 was dispensable for innate immunity to OPC.

MATERIALS AND METHODS

Mice. Mice were on the C57BL/6 background and age and sex matched. IL-23p19$^{-/-}$ mice were kindly provided by Genentech (S. San Francisco, CA), and IL-17RA$^{-/-}$ mice were from Amgen (Seattle, WA). CARD9$^{-/-}$ mice were from Xin Lin, MD Anderson Cancer Center (22). All other mice were from the Jackson Laboratory (Bar Habor, ME). Mice were inoculated sublingually with a 2.5-mg cotton ball soaked in 10^7 CFU *C. albicans* (strain CAF2-1) for 75 min under anesthesia, as described in detail previously (18, 20). No antibiotics or other drugs were administered before or during the infections, and mice were weighed daily. At the indicated times after infection (ranging from 1 day to 6 weeks), tongue tissue was processed in a GentleMACS Dissociator (Miltenyi) and homogenates were plated in serial dilutions on yeast extract-peptone-dextrose–AMP

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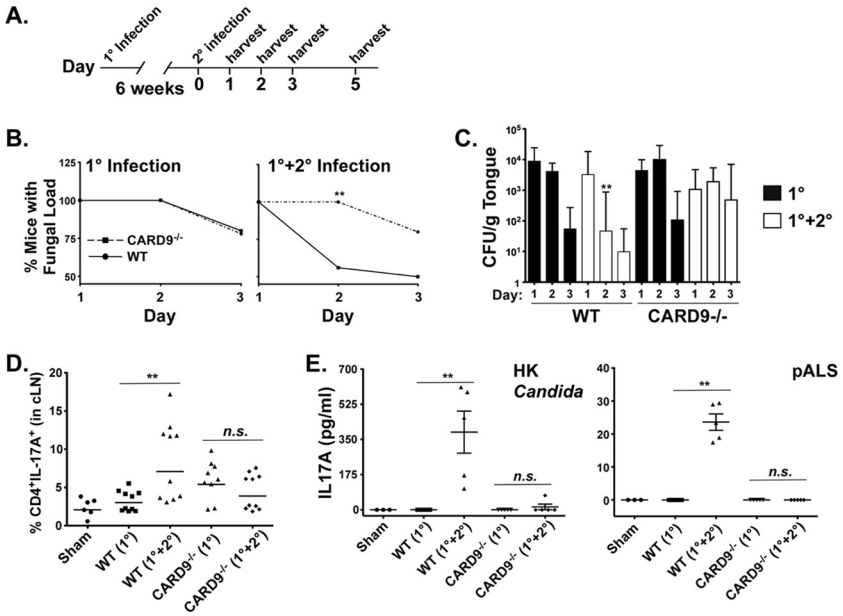

FIG 1 CARD9 is required for adaptive Th17 responses to OPC. (A) Schematic diagram of OPC model. (B) CARD9$^{-/-}$ mice fail to show enhanced clearance of *C. albicans* from the oral mucosa following rechallenge. CARD9$^{-/-}$ or WT mice were subjected to a 1° or 1°+2° oral inoculation with *C. albicans*, and fungal load in tongue was assessed at days 1 and 2 postinfection. The percentage of mice with a detectable fungal count at each time point is shown. **, $P < 0.01$ (by chi-square analysis). (C) CARD9$^{-/-}$ mice show increased fungal loads in the oral cavity following rechallenge. Data represent fungal loads in WT and CARD9$^{-/-}$ mice at days 1 to 3 postinfection during 1° infection or 1°+2° infection. Data are expressed as CFU/g tongue tissue. Error bars show geometric mean with 95% confidence interval (CI). **, $P < 0.01$ (by *t* test with Mann-Whitney correction). (D) CARD9$^{-/-}$ mice exhibit impaired Th17 cell frequencies in cLN. CD3$^+$ CD4$^+$ cells from cLN of infected WT or CARD9$^{-/-}$ mice were analyzed for intracellular expression of IL-17A, and the percentage of total LN cells is indicated. **, $P < 0.01$ (by *t* test with Mann-Whitney correction). (E) CARD9$^{-/-}$ mice have reduced *Candida*-specific Th17 responses following rechallenge. Total cLN cells from CARD9$^{-/-}$ or WT mice subjected to the indicated conditions were cultured *in vitro* for 5 days with HK *Candida* extract or pALS peptide. Supernatants were analyzed in triplicate for IL-17A. **, $P < 0.01$ (by *t* test with Mann-Whitney correction). n.s., not significant.

(YPD-AMP) agar for colony enumeration in triplicate. For rechallenge experiments, mice were given a primary infection with *C. albicans* or a sham treatment with the cotton ball soaked in phosphate-buffered saline (PBS); after 6 weeks, all mice were reinfected with *C. albicans*, as described previously (18). All mice were swabbed orally and the samples plated onto YPD-AMP before each inoculation to assess oral yeast colonization. Data were analyzed on GraphPad Prism using Student's *t* test with Mann-Whitney correction or chi-square analyses, as appropriate. Protocols were approved by the University of Pittsburgh IACUC. All experiments were performed a minimum of two times.

***C. albicans* culture, fluorescence-activated cell sorting (FACS), and T cell stimulation.** *C. albicans* (strain CAF2-1) was cultured in YPD at 30°C overnight with agitation. A heat-killed (HK) extract was prepared with 1 ml (~4×10^8 cells) culture boiled for 45 min. ALS1/3 peptide (KGLNDWNYPVSSESFSYT) was from Biosynthesis (Lewisville, TX), as described previously (18). Cervical lymph node (cLN) stimulation was performed by plating 10^6 cLN cells per well with 2×10^6 HK *C. albicans* or 10 μg/ml ALS1/3 peptide diluted in serum-free AIM V media (Gibco, Invitrogen) with 20 U/ml IL-2. Supernatants were analyzed in triplicate for IL-17 and gamma interferon (IFN-γ) by enzyme-linked immunosorbent assay (ELISA) (eBiosciences). Intracellular IL-17 and IFN-γ were detected with anti-IL-17–phycoerythrin (anti-IL-17-PE) and anti-IFN-γ–allophycocyanin. Dead cell exclusion was performed using a Live/Dead Violet Viability/Vitality Dye kit (Molecular Probes/Invitrogen). Intracellular cytokine staining was performed with a Cytofix Cytoperm kit (BD Biosciences). Data were acquired on an LSR II instrument and analyzed by Flowjo.

RNA and quantitative PCR (qPCR). Frozen tongue was homogenized in RLT buffer (RNAeasy kit; Qiagen, Valencia, CA) with a GentleMACS Dissociator (M-tubes, RNA02 program; Miltenyi). cDNA was synthesized with a SuperScript III first-strand synthesis system (Invitrogen). Quantification was determined by real-time PCR with SYBR green (Quanta BioSciences, Gaithersburg, MD) on a 7300 real-time PCR system (Applied Biosystems, Carlsbad, CA) normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Primers were from SA Biosciences (Qiagen).

RESULTS

CARD9 is necessary for adaptive responses to OPC. Although CARD9 is linked to Th17 generation in the context of disseminated candidiasis, its role in mucosal candidiasis has not been defined, with studies in humans limited to a single family (14). Moreover, immunity in the oral mucosa is distinct from immunity in the intestinal tract in many respects; for example, Dectin-1-deficient mice are resistant to OPC (23), and yet Dectin-1 contributes to gastric inflammation induced by *C. albicans* in mice (17, 24). Here, we addressed the role of CARD9 in adaptive immunity to OPC using a rechallenge model in which mice are subjected to a primary oral infection with *C. albicans* followed by rechallenge after 6 weeks (Fig. 1A) (18). In wild-type (WT) mice, a strong CD4$^+$ Th17 cell response occurs in the cLN, causing accelerated clearance of *C. albicans* from the oral mucosa (18). Because CARD9 promotes Th17 differentiation in response to fungi, we hypothesized that CARD9$^{-/-}$ mice would not be able to mount an effective adaptive response in OPC. Accordingly, WT and CARD9$^{-/-}$ mice (22) were subjected to a primary infection only (1°) or to a primary infection followed by rechallenge (1°+2°), as described previously (18). Fungal load in the tongue was assessed at days 1, 2, and 3 postinfection. In animals receiving only a 1° exposure, the percentages of WT and CARD9 mice with

detectable *C. albicans* colonization were identical (Fig. 1B, left). In the 1°+2° infection setting, significantly more WT mice fully cleared *Candida* after rechallenge than CARD9$^{-/-}$ mice (Fig. 1B, right). Consistently, WT mice showed a reduced fungal burden in the 1°+2° setting at days 2 and 3 postinfection, while CARD9$^{-/-}$ mice showed higher overall fungal loads (Fig. 1C). Therefore, CARD9$^{-/-}$ mice do not exhibit the additional protection provided by prior exposure to *Candida* that occurs in WT mice.

WT mice subjected to rechallenge mount a strong CD4^{+} IL-17^{+} T cell response in the draining cervical LN (cLN) following stimulation with *Candida* antigens. To determine whether this response occurs in CARD9$^{-/-}$ mice, cells from WT or CARD9$^{-/-}$ cLN were stimulated with phorbol myristate acetate (PMA) and ionomycin and stained for CD3 (not shown), CD4, and IL-17A. As seen previously (18), there was no Th17 response in WT mice receiving only a primary challenge but a strong response was seen in mice receiving a 1°+2° infection. In CARD9$^{-/-}$ mice, there was no Th17 response in either the 1° or 1°+2° infection settings (Fig. 1D), thus demonstrating that the impaired adaptive response to *C. albicans* is associated with impaired induction of Th17 cells. There was no induction of Th1 cells in either the WT or CARD9$^{-/-}$ mice (data not shown). To determine whether CARD9 deficiency impaired *Candida*-specific Th17 responses, cLN cells were cocultured with a heat-killed (HK) *C. albicans* extract or an major histocompatibility complex class II (MHC-II)-restricted peptide derived from a *C. albicans* cell wall adhesin (pALS) (19). WT mice secreted IL-17A in response to pALS and HK *C. albicans* (Fig. 1E), but CARD9$^{-/-}$ mice showed severely impaired *C. albicans*-specific responses (Fig. 1E). These results indicate that CARD9 is necessary for induction of *C. albicans*-specific Th17 cells that are needed to mediate a recall response to OPC.

CARD9 is redundant for innate immunity to OPC. Adaptive immunity to fungi is triggered by signals from the innate immune response. In its capacity as an adaptor for CLRs, CARD9 mediates induction of Th17-polarizing cytokines in APCs. To test the hypothesis that the impaired adaptive response in CARD9$^{-/-}$ mice was a consequence of impaired innate immunity, we subjected CARD9$^{-/-}$ mice to an acute 5-day model of OPC in which the response was driven by the innate immune system. As we previously reported (20), IL-12$^{-/-}$ mice were resistant to OPC, whereas IL-23$^{-/-}$ and IL-17RA$^{-/-}$ mice were profoundly susceptible (Fig. 2A). In contrast, CARD9$^{-/-}$ mice were largely, albeit not completely, resistant to OPC. The majority of the mice (66%) showed no detectable fungal burden whatsoever (Fig. 2A). Some mice still had fungal colonization at day 5 (33%) but at a level that was only 11% of that in the IL-23$^{-/-}$ mice. Moreover, WT and CARD9$^{-/-}$ mice fully regained weight by day 5, whereas IL-23$^{-/-}$ mice showed sustained weight loss, indicative of overt disease (Fig. 2B).

We then asked whether some CARD9$^{-/-}$ mice remained persistently infected or simply showed delayed clearance. IL-17RA$^{-/-}$ mice exhibited a fungal load throughout the 5-day experimental period, as we observed previously (20) (Fig. 2C). In contrast, CARD9$^{-/-}$ mice began to clear *C. albicans* by day 3 in a pattern similar to that seen with the WT (Fig. 2C; see also Fig. 1C). On day 12, many of the CARD9$^{-/-}$ mice still showed a fungal load (Fig. 2D, left), and 22% of CARD9$^{-/-}$ mice still had oral *Candida* colonization even after 6 weeks (Fig. 2D, right). These data show that fungal clearance is impaired only mildly in CARD9$^{-/-}$ mice and, notably, to a much lesser degree than in IL-17RA$^{-/-}$ or IL-23$^{-/-}$ mice.

To determine whether loss of CARD9 impacted IL-17 production or signaling during OPC, we evaluated mRNA expression of *il17a*, IL-17-dependent genes (*il6*, *lcn2*), and Th17 polarizing cytokine genes (*il1b*, *il23*) in tongue. There was reduced, although not abrogated, *il17a* expression in CARD9$^{-/-}$ mice at day 1; since *il17a* is shut off in WT mice rapidly after day 2 of infection, there were no differences after this time point (Fig. 3A). Consistently, expression of *il6* and *24p3*, IL-17 target genes, was reduced in CARD9$^{-/-}$ mice compared to WT mice at day 1, although this did not reach statistical significance for *lcn2* (Fig. 3B). In terms of Th17 inductive genes, expression of *il1b* was similarly reduced (Fig. 3C). Although there was not a significant difference with respect to expression of *il23*, there was a trend to reduced expression (Fig. 3C). These data indicate that CARD9 regulates expression of Th17-related genes at early time points postinfection, probably accounting for the partial susceptibility seen in the CARD9-deficient mice.

CARD9 is dispensable for control of *C. albicans* in the GI tract. Although humans with defects in the Th17/IL-17 axis or Dectin-1 and CARD9 are susceptible to CMC, only patients with mutations in CARD9 exhibit disseminated disease (14). Moreover, the *CARD9* locus is a susceptibility allele for inflammatory bowel disease (25), suggesting that CARD9 could be important for maintaining barrier immunity to *C. albicans* at GI surfaces. To address this possibility, we evaluated fungal dissemination in the 5-day OPC model. WT mice did not exhibit fungal dissemination (Fig. 4A), while WT mice treated with cortisone or IL-23$^{-/-}$ mice demonstrated fungal loads in kidney, liver, spleen, and brain (Fig. 4B and C). Despite fungal burden throughout the GI tract, CARD9$^{-/-}$ mice did not exhibit dissemination to visceral organs (Fig. 4D). Thus, CARD9 is redundant for the control of *C. albicans* in the GI tract.

DISCUSSION

CARD9 has emerged as a central signaling intermediate downstream of CLRs, analogous to MyD88 in the Toll-like receptor pathway (26). Both CARD9 and CLRs are linked to immunity to *C. albicans*, but most studies to date have focused on models of systemic candidemia. *Candida* is a dimorphic fungus with distinct recognition requirements in its yeast form versus its hyphal form. Whereas disseminated candidiasis is mainly induced by a yeast form of *C. albicans*, mucosal disease is more strongly characterized by invasive hyphae and pseudohyphae (27). In addition, while CARD9 drives differentiation of conventional Th17 cells in the adaptive response, it has become clear recently that the importance of innate sources of IL-17 is equal to if not greater than that of conventional Th17 cells in some settings (28). For all these reasons, we were prompted to investigate the role of CARD9 in the most common form of mucosal candidiasis, OPC. In this report, we made the unexpected finding that CARD9 is largely dispensable for the innate immune response to OPC whereas it is clearly vital for the adaptive Th17 response.

Immunity to oral candidiasis is strongly dependent on signals from IL-17, since mice and humans with defects in IL-17RA, IL-17RC, and Act1 (the major adaptor for the IL-17 pathway [29]) are exquisitely susceptible to CMC (6, 20, 21, 30). Similarly, humans with neutralizing antibodies (Abs) against Th17 cytokines arising from autoimmune polyendocrinopathy syndrome-1

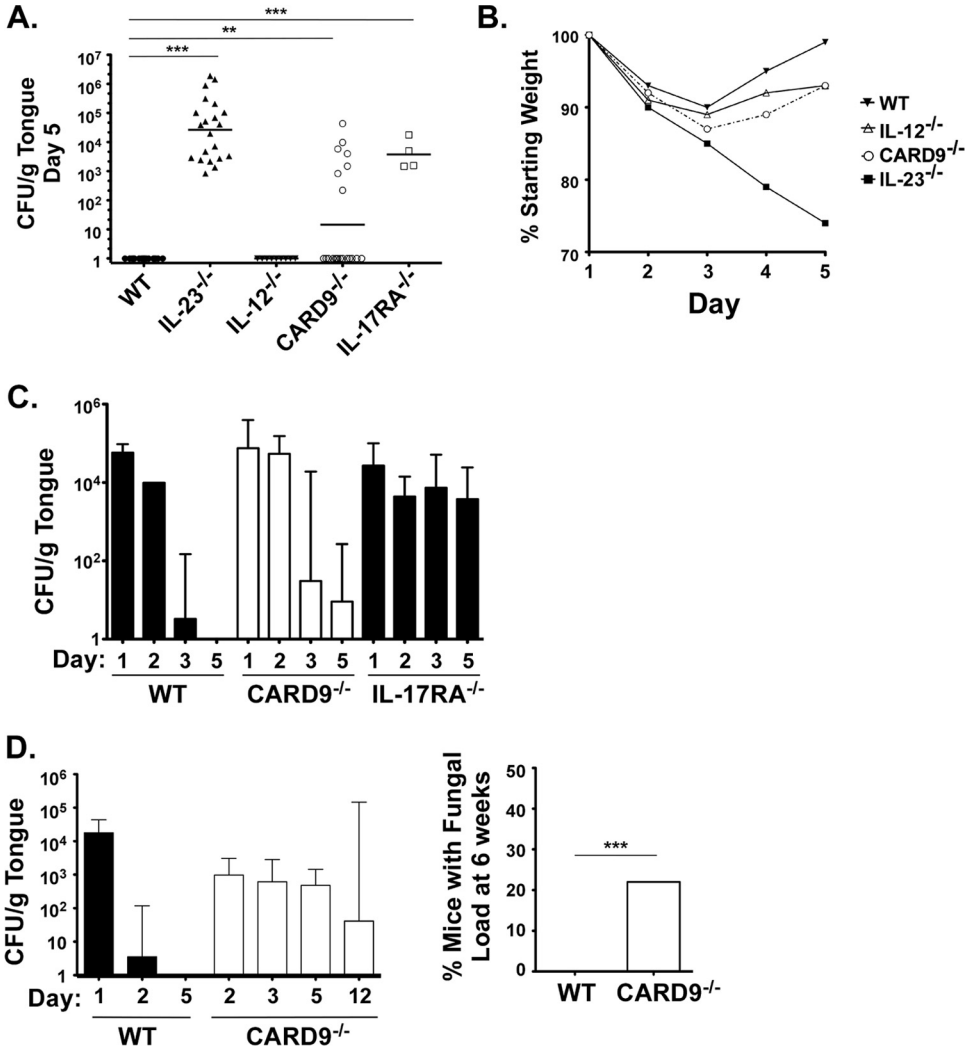

FIG 2 CARD9 is dispensable for immunity to acute OPC. (A) CARD9$^{-/-}$ mice show only mild susceptibility to acute *C. albicans* oral infection. The indicated mice were subjected to OPC for 5 days, and fungal load in tongue was assessed. Data are expressed as CFU/g tongue tissue. **, $P < 0.01$; ***, $P < 0.005$ (by *t* test with Mann-Whitney correction). (B) CARD9$^{-/-}$ mice regain weight following oral infection with *C. albicans*. The indicated mice were subjected to OPC and weighed daily for 5 days. Data are expressed as the average percentage of starting weight on day 1. (C) IL-17RA signaling but not CARD9 is required for clearance of *C. albicans* from the oral cavity. The indicated mice were subjected to infection with *C. albicans*, and fungal load in tongue was assessed at days 1 to 5 postinfection. Data are expressed as CFU/g tongue tissue. Error bars show geometric mean with 95% CI. (D) CARD9$^{-/-}$ mice exhibit delayed clearance of fungi from the oral mucosa. (Left) WT or CARD9$^{-/-}$ mice were subjected to oral infection with *C. albicans*, and fungal burden in tongue was assessed at the indicated days postinfection. Data are expressed as CFU/g tongue tissue. Error bars show geometric mean with 95% CI. (Right) At 6 weeks postinfection, an oral swab was plated onto YPD-AMP agar, and the percentage of mice with evidence for *C. albicans* colonization is indicated. ***, $P < 0.01$ (by *t* test with Mann-Whitney correction).

(*AIRE* deficiency) or certain thymomas are also prone to mucosal candidiasis (31–33). It is usually assumed that conventional Th17 cells are the dominant source of IL-17, as individuals with defects in generation of Th17 cells such as STAT3 experience CMC (34). However, in the standard 5-day mouse model of OPC used here, clearance from the oral cavity occurs within 3 to 4 days, long before an adaptive immune response can be induced (Fig. 1 and 2) (20, 35). We and others previously confirmed that there is no preexisting adaptive immunity to *C. albicans* in the CD4$^+$ T cell compartment, and thus this mouse model provides the opportunity to evaluate both innate and adaptive immunity, using acute infection and a rechallenge model (18, 19).

Our finding that CARD9 is required almost exclusively for the adaptive and not the innate response in mice may seem at first to be in conflict with the observation that CARD9-deficient humans are susceptible to CMC (14). However, it is likely that the relative contributions of innate versus adaptive IL-17 responses in humans are different from those in mice. That is, conventional Th17 responses appear to be critical in humans as evidenced by the high incidence of OPC in AIDS (1, 3, 36). Mice, in contrast, mount more powerful oral innate responses, perhaps associated with differing diets or exposure to oral microbes (e.g., mice are coprophagic). In addition, whereas mice with impaired TLR signaling exhibit a broad susceptibility to viral, bacterial, and fungal pathogens, humans with impaired TLR signaling (IRAK4 deficiency and MyD88 deficiency) exhibit a relatively narrow range of

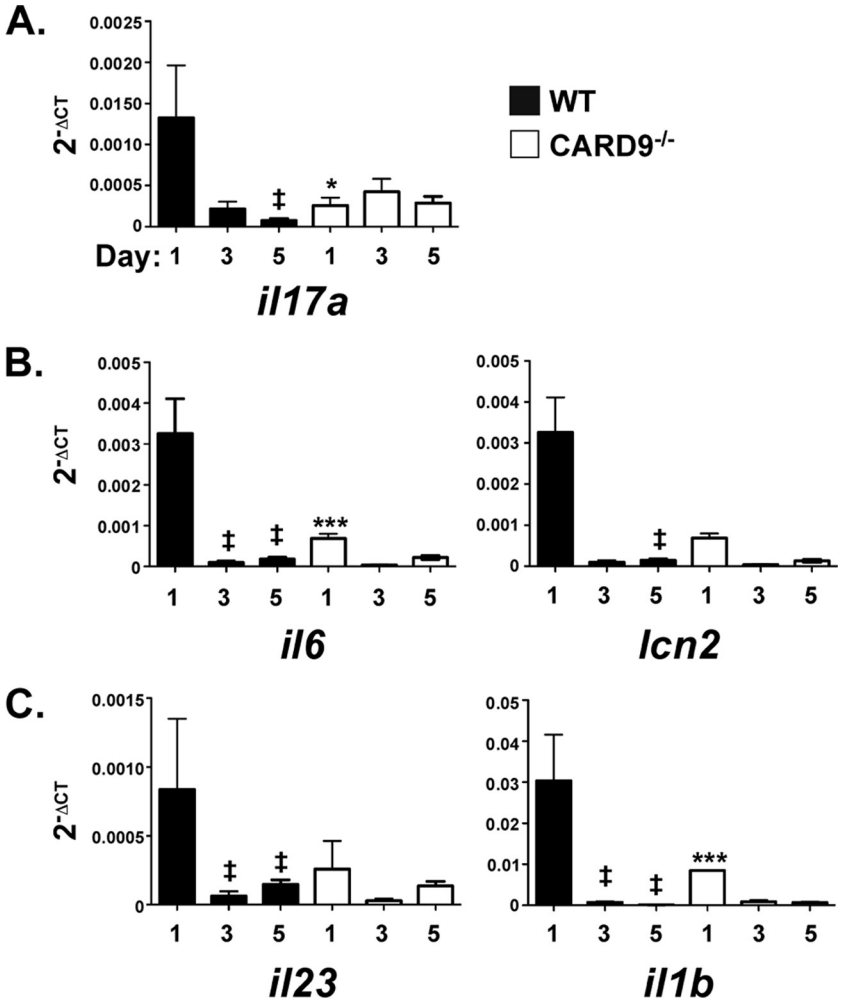

FIG 3 Reduced IL-17 signature gene expression in CARD9$^{-/-}$ mice following OPC. Tongue tissue from WT or CARD9$^{-/-}$ mice at day 1 or day 3 postinfection was analyzed for expression of *il17a* (A), IL-17 target genes *il6* and *lcn2* (encoding lipocalin 2, 24p3) (B), and Th17 inductive cytokines *il23* and *il1b* (C). Expression was normalized to GAPDH. n.s., not significant. ‡, $P < 0.05$ (compared to WT mice at day 1); *, $P < 0.05$; ***, $P < 0.001$ (compared to WT mice at the matched time points by analysis of variance [ANOVA] with *post hoc* Tukey's test).

susceptibility to pyogenic bacteria. Moreover, the incidence of infection decreases with the age of the patient, suggesting that adaptive responses may compensate for innate defects in humans chronically exposed to pathogens (37–39).

Since CARD9 signaling is not required for effective innate immune responses to OPC, what is the central pattern recognition receptor (PRR) pathway? Dectin-1 was shown to be dispensable in an OPC mouse model similar to the one used here (23), although a study in gastric candidiasis indicated a dependence on Dectin-1 only in certain mouse strains (including C57BL/6, used in this report) (24). Mincle is another candidate CLR required for protection from systemic candidiasis (40), but if Mincle plays a role in OPC it does so in a CARD9-independent manner. We found that neutralizing Abs against Dectin-2 and Dectin-3 did not increase susceptibility to OPC, consistent with the preserved immunity in CARD9$^{-/-}$ mice (S.B. and N.H.-S., unpublished observations). Elegant studies by Hise et al. demonstrated nonredundant roles of NLRP3 and NLRC4 inflammasome complexes and TLR2 in immunity to OPC (23, 41). Therefore, CLRs appear to be most important for adaptive immunity to *C. albicans*.

OPC does not typically progress to disseminated disease. For example, HIV/AIDS patients with low CD4 counts are exquisitely susceptible to OPC but do not experience systemic candidiasis (1, 42). Rather, the source of *C. albicans* in invasive infections is thought to be the GI tract, as two important risk factors for invasive candidiasis are exposure to broad-spectrum antibiotics and GI tract surgery (2). However, the mechanisms that maintain barrier immunity in mucosal candidiasis remain poorly understood. Our data indicate that CARD9 is not essential for maintenance of barrier immunity in the gut, whereas IL-23 is more critical (Fig. 4). This dichotomy reflects the idea that IL-23 is required *in vivo* not only for conventional adaptive Th17 responses but also for all known innate IL-17-producing cells (28). However, even though IL-23-deficient mice showed some dissemination to visceral organs, all the mice survived infection, in some cases for as long as 6 weeks postinfection (N.H.-S., unpublished data). The same is true for IL-17RA-deficient mice (20).

To date, there have been no vaccines available for protection against *C. albicans* or other fungi (43). Understanding the requirements for innate immunity to mucosal candidiasis will be key for

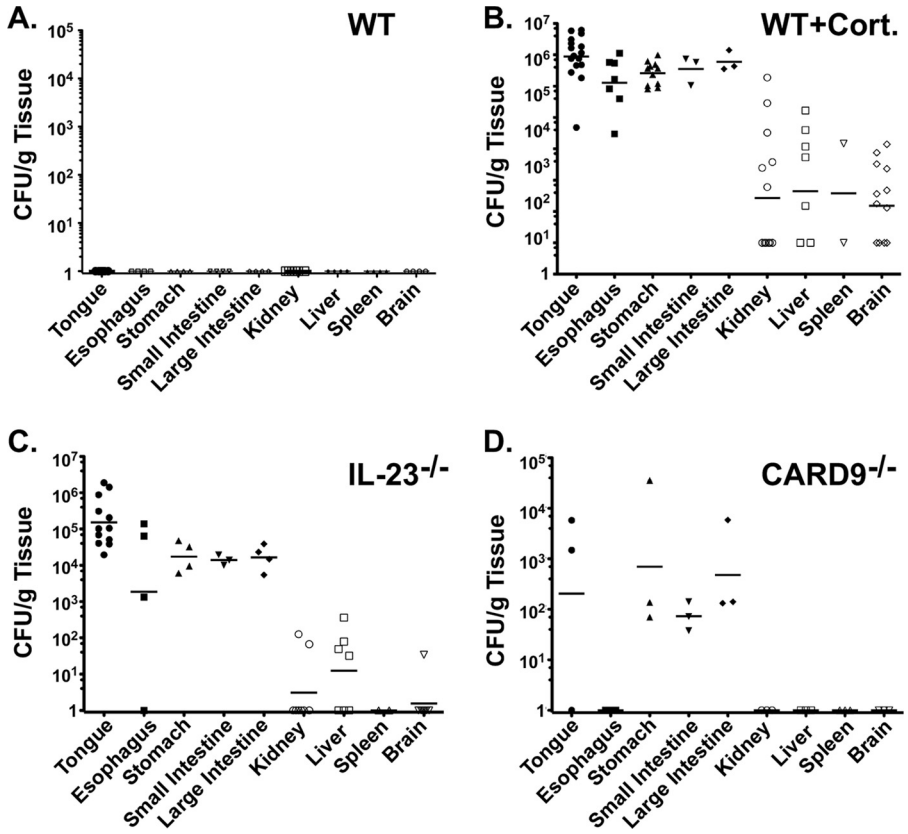

FIG 4 CARD9$^{-/-}$ mice do not exhibit fungal dissemination from the GI tract following OPC. The indicated mice were subjected to OPC for 5 days. In the experiments whose results are shown in panel B, cortisone acetate (Cort.) was administered to WT mice at days −1, +1, and + 3 postinoculation at 225 mg/kg of body weight. The indicated organs were analyzed for *C. albicans* colonization. Bars show geometric means.

generating effective vaccines or therapies for individuals with defective adaptive immune responses, as in infants, AIDS patients, or patients undergoing chemotherapy and irradiation.

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